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Patterns of testosterone in three Nearctic-Neotropical migratory songbirds during spring passage

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Abstract

Preparation for breeding may overlap extensively with vernal migration in long-distance migratory songbirds. Testosterone plays a central role in mediating this transition into breeding condition by facilitating changes to physiology and behavior. While changes in testosterone levels are well studied in captive migrants, these changes are less well known in free-living birds. We examined testosterone levels in free-living Nearctic-Neotropical migrants of three species during their vernal migration. Testosterone levels increased during the migratory period in males of all three species but significantly so in only two. Testosterone levels in females remained the same throughout their migration. Our results support the extensive overlap between vernal migration and breeding preparation in male songbirds. The pattern of testosterone changes during vernal migration is far from clear in females.

Keywords: testosterone, migration, birds, breeding preparation, annual cycle

1. Introduction

Animals express different phenotypes at different times of the year as they transition through their annual cycle. Piersma and van Gils (2011) purport that life-history stages are the specific phenotypic periods that exist within a single individual at different points during the annual cycle. Finite State Machine Theory posits that there is a limit to the number of behavioral, physiological, and morphological traits that can be expressed within an individual at any time (Jacobs and Wingfield, 2000). Accordingly physiological tradeoffs must occur as an organism transitions between states, i.e. stages in the annual cycle (Jacobs and Wingfield, 2000; Ramenofsky, 2011). Migratory songbirds typically exhibit the following series of annual life-history stages: breeding, pre-basic molt, autumn migration, overwintering, and vernal migration; and some species also complete a pre-alternate molt prior to vernal migration (sensu Jacobs and Wingfield, 2000). The expression of traits associated with these different life-history stages of migratory species must coincide due to temporal constraints, i.e. the addition of the two lengthy migratory periods necessitates overlap between the migratory stage and the previous and subsequent stages. If we are to understand the biology of migratory species we must understand how these life-history stages interact with one another (Greenberg and Marra, 2005).

Hormones mediate many of the physiological and behavioral changes as individuals transition between life-history stages (Wingfield, 2008). For example, the termination of breeding and the onset of pre-basic molt is promoted by prolactin (Dawson and Sharp, 1998; Dawson, 2006). Numerous hormones regulate a variety of physical and behavioral traits during breeding, so it is critical to gain a complete understanding of the hormonal changes that take place as seasonally-breeding birds prepare for reproduction. Preparation for breeding in migratory songbirds may be particularly intricate because these birds are balancing the energetic and physiological constraints they experience in two successive and particularly demanding phases of the annual cycle, migration and breeding. While it is clear that events occurring in one life history stage influence survival and reproductive success in subsequent stages (Paxton and Moore, 2015; Studds et al., 2008; Tonra et al., 2011a), studies focusing on the physiological overlap between stages in free-living migrants are limited and their results are inconsistent. For example, while Tonra et al. (2013) detected increases in androgens in American Redstarts (*Setophaga ruticilla)* on the wintering grounds prior to vernal migration and Wingfield and Farner (1978a, 1978b) showed that testosterone increased during vernal migration in White-crowned Sparrows (*Zonotrichia leucophrys*), Bauchinger et al. (2007) did not detect any increase in migrating Garden Warblers (*Sylvia borin*).

Testosterone (T) is a steroid hormone that mediates physiology and behavior throughout the annual cycle. T plays a central role during reproduction and may be important in facilitating the transition between breeding and the stages preceding it (Ramenofsky and Wingfield, 2006). In seasonally-reproducing birds, breeding preparation involves photostimulation and subsequent recrudescence of the hypothalamic-pituitary-gonadal (HPG) axis (Hahn et al., 2009). T is present in both males and females at varying levels throughout the annual cycle (Ketterson et al., 2005) and plays a primary role in the expression of breeding behaviors in both sexes (Balthazart, 1983; Wolfgang Goymann and Wingfield, 2014; Rosvall, 2013; Staub and De Beer, 1997; Wingfield et al., 2001). Testosterone is also a precursor to the production of estradiol in females, which is required for ova development (Norris, 1997). Since physiological breeding preparation takes approximately one month to complete (Ramenofsky, 2011), this process necessarily overlaps with the previous life-history stage and testosterone levels may increase well before the breeding season begins (Tonra et al., 2013; Wingfield and Farner, 1978a, 1978b; see Wingfield et al., 1990).

In addition to its influence during the breeding season, T plays a role during vernal migration. Almost a century ago, Rowan (1925) showed that T is required for birds to develop normal migratory behaviors. Many subsequent studies have also revealed that T and/or other gonadal hormones influences the expression of various migratory traits including migratory restlessness, hyperphagia, fat deposition, and accompanying mass gains (Deviche, 1995; King and Farner, 1962; Morton and Mewaldt, 1962; Schwabl and Farner, 1989a, 1989b; Schwabl et al., 1988; Stetson and Erickson, 1972; Weise, 1967). Further, the schedule for the expression of these migratory traits is advanced when T levels are experimentally elevated (Owen et al., 2014; Tonra et al., 2013, 2011b). These studies indicate that T may influence physiological aspects of migration related to energetic condition (fattening, mass gains) and given T’s influence on aggression and territoriality during the breeding season (Balthazart, 1983; Goymann and Wingfield, 2014) we might expect a link between T and resource competition during migration. While elevated T leads to increases in activity and food intake in captive birds (Wikelski et al., 1999) and to faster movement rates and more time spent foraging in free-living breeding birds (Lynn et al., 2000), T's influence on competition during migration has yet to be explored.

This study investigates variation in T for long-distance migrants as they progress towards their breeding grounds. To do so, we measured T levels in three Nearctic-Neotropical passage migrants at a southern and a northern site during vernal migration thus representing "far" and "near" relative distances to the breeding grounds. Our major hypothesis was that T increases as birds move closer to their breeding grounds during vernal migration. We tested the following specific predictions: (1) birds sampled at our northern site would have higher circulating T when compared to conspecifics sampled at our southern site; (2) males will have higher circulating T than females; (3) T and energetic condition would be positively correlated; and (4) T and the potential for competitive interactions would be correlated. In addition, we investigated the potential for an interaction between corticosterone (CORT) and T since some studies have indicated that CORT may suppress T (Deviche et al., 2001; Swett and Breuner, 2008).

2. Materials and Methods

2.1. Study Species

The focal species of this study were three boreal-breeding Nearctic-Neotropical passerines: Swainson's Thrush (SWTH; *Catharus ustulatus*), Northern Waterthrush (NOWA; *Parkesia noveboracensis*), and Magnolia Warbler (MAWA; *Setophaga magnolia*). All three winter in Central and/or South America and breed primarily in boreal regions of North America, with the southern-most breeding in Eastern North America around 39 degrees North latitude (Poole, 2005; Fig. 1). These species do not winter or breed at either of our study locations and thus are transient migrants at both.

2.2. Capture and Sampling

We passively captured migrants at two study locations that represent “far” and “near” relative distances to their breeding ranges (Fig. 1). Our southern, “far”, location was Johnson’s Bayou in Cameron Parish, Louisiana (29° 45’ N 93° 30’ W; hereafter “Louisiana”) where we operated up to 29 mist nets during April and May 2011-2014. This site is approximately two hectares in size but is located within a larger chenier forest extending approximately 20 miles along the northern coast of the Gulf of Mexico in southwest Louisiana. Our northern, “near”, location was Appledore Island in York County, Maine (42°58’N, 70°36’W; hereafter “Maine”) where we operated up to 10 mist nets during May and June 2011-2014. This site is a 33-hectare island located in the Isles of Shoals archipelago and is approximately 9.5 km from the nearest point on the mainland. We checked nets at both locations at least every 20-30 minutes.

We obtained a blood sample via brachial puncture from each individual within 10 minutes of extraction from a net (mean ± SD; 4.5 ± 2.0 min). We used either a 26- or 27-gauge needle, depending on the species, and collected blood into heparinized capillary tubes. Samples were placed on ice or in a refrigerator until centrifuged later that same day at 14,000 rpm for 10 minutes. Plasma was extracted and stored at -20°C until analyzed. Red blood cells were placed in approximately 500 µl of lysis buffer (50mM TRIS, 10mM EDTA, 1% SDS, 0.1 M NaCl) and then stored at either -20°C or 4°C. These red blood cells were used to determine the sex of individuals genetically because neither NOWA nor SWTH exhibit extensive sexual dimorphism.

Each bird was banded with a USGS aluminum leg band and measurements of wing length (unflattened wing chord; nearest 0.5 mm), cloacal diameter (nearest 0.1 mm), and body mass (nearest 0.01g) were taken. Age was determined according to Pyle (1997) as either second-year (SY) or after-second-year (ASY). Occasionally we recorded a bird as the less specific age class of after-hatching-year (AHY) if we were unable to differentiate further. For MAWA, sex was determined based on plumage characteristics (Pyle, 1997). Subcutaneous fat deposits were assessed to quantify energetic condition, according to Helms and Drury (1960). Sample sizes by species, location, age, and sex are provide in Table 1.

2.3. Plasma Testosterone Assays

Plasma T was determined with an enzyme immunoassay (EIA; Assay Designs, Inc. #901-065; Jawor, 2007; Jawor et al., 2007). Depending on sample volume, 20-40 µl of plasma was used and 2000 cpm of H3 labeled T (PerkinElmer) was added to each plasma sample to allow calculation of recoveries after three extractions with diethyl ether. Extracts were re-suspended in 50 µl of ethanol and 300 µl of the assay buffer provided in the EIA kit. Recoveries were determined with 100 µl from each reconstituted sample. Samples were run in duplicate with 100 µl of each reconstituted sample in the EIA. Testosterone concentrations were determined using a logistic curve (Microplate Manager; Bio-Rad Laboratories, Inc., Hercules, California) and corrected for incomplete recoveries and initial plasma volume. Intra- and inter-assay variations were determined based on three standard samples of known T concentration placed in each assay plate. Intra-assay variation ranged from 2% to 13% and inter-assay variation was 13.8%.

2.4. Plasma Corticosterone Assays

Given the possibility that CORT may suppress T (Deviche et al., 2001; Swett and Breuner, 2008), we determined CORT levels in a subset of our samples, dependent on adequate plasma volume. Plasma CORT was determined with an enzyme immunoassay (EIA; Arbor Assays Inc. #K014; DeVries and Jawor, 2013). Ten µl of plasma were used with 2000 cpm of H3 labeled CORT (PerkinElmer) to allow calculation of recoveries after three extractions with diethyl ether. Extracts were re-suspended in 400 µl of the assay buffer provided in the EIA kit. Recoveries were determined with 100 µl from each reconstituted sample. Samples were run in duplicate with 50 µl of each reconstituted sample on the EIA. CORT concentrations were determined using a logistic curve (Microplate Manager; Bio-Rad Laboratories, Inc., Hercules, California) and corrected for incomplete recoveries and initial plasma volume. Intra- and inter-assay variations were determined based on four samples from a plasma pool placed in each assay plate. Intra-assay variation ranged from 6% to9% and inter-array variation was 6%.

2.5. Genetic Sex Determination

We extracted DNA using a DNeasy Tissue Kit, #69506, Qiagen, Valencia, CA, following standard protocol from Qiagen for nucleated erythrocytes. Polymerase chain reaction was used to amplify the Chromobox-Helicase-DNA binding gene, different versions of which are found on the Z and W chromosomes of birds, using the P2 and P8 primers (Griffiths et al., 1996, 1998). PCR products were run on an agarose gel where a single band at 325 bp indicated male and two bands (one each at 325 and 375 bp) indicated female.

2.6. Statistical Analyses

Prior to analyses, T and CORT data were corrected for inter-assay variation based on the standards within each assay. Further, both T and CORT failed to meet normality assumptions. Natural log (NOWA and MAWA T), square root (SWTH and NOWA CORT), or sixth root (SWTH T) transformations corrected the disparity, and thus transformed data were used in all analyses. Also, birds aged as unknown/AHY were excluded from all analyses that included age.

We calculated energetic condition for each bird by subtracting the species- and size-specific fat-free mass from the individual’s body mass, thus larger values of energetic condition are indicative of birds with larger fat stores, after correcting for size (Ellegren, 1992; Owen and Moore, 2006). For this calculation we used the combined long-term data sets from both study sites, Louisiana (1993-2010) and Maine (1990-2009), for each of the three species (SWTH: N = 1,877; NOWA: N = 2,265; MAWA: N = 6,011). For each species and wing chord, we regressed body mass on fat score and then used the resulting intercept in a final regression of fat-free mass on wing chord. The resulting equations solve for fat-free mass (y) where ‘x’ represents wing chord (SWTH: y = 0.20x + 5.8; NOWA: y = 0.09x + 7.9; MAWA: y = 0.09x + 2.6).

All analyses were performed in Program R (version 3.1.2). We used linear mixed effects models to allow the inclusion of random effects (packages lme4 and lmerTest; Bates, 2010; Bates et al., 2014). We selected the best model for each set of analyses using likelihood ratio tests by comparing full models (will all possible interactions) to simpler models, which allowed detection of significant interactions and first-order effects. We evaluated significance for main effects using likelihood ratio tests that compared a model with the variable in question to the null model. If necessary, we evaluated pairwise effects using least squares means tests with Tukey correction (package lsmeans in R).

We examined T in each study species in a series of analyses. First, we investigated whether circulating T was influenced by a variety of factors measured in this study that should be considered when testing our other predictions. These factors included date (day of year), the latency between extraction from nets and blood sampling (handling time), energetic condition, and time of day (calculated as sampling time less the time of local sunrise). Analyzing these factors first allowed us to identify which variables should be included as random variables in subsequent analyses and thus in this first set analyses, age, sex, year, and location were set as random effects. Second, we analyzed whether variation in circulating T levels can be explained by age, sex, or location of sampling (far or near the breeding grounds). In this second set of analyses, year and any effects of date, handling time, time of day, and energetic condition that were supported by model selection from the first analysis were kept as separate random effects. Third, we examined whether T was correlated with two measures of competition a migrant would experience on the day of sampling. For this analysis we compared circulating T to both the total number of other migrants captured and the number of migrants captured within each species’ foraging group. MAWA, NOWA and SWTH were considered to be upper canopy, ground, and ground/midlevel foragers respectively. All species captured at our two study locations (# species = 98, # migrants = 21,050) were broadly designated as ground/understory, midlevel/lower-canopy, and/or upper canopy foraging guilds (loosely based on De Graaf et al., 1985). Since it is likely that site-specific factors influence these measures of competition, this third set of analyses were run for each site separately and all aforementioned variables that were supported by the model selection in our first and second sets of analyses were included as random effects.

In a separate analysis, we examined the potential influence of CORT on circulating T on a subset of sampled birds. Because of their small body size we were unable to obtain enough blood from MAWA for this additional hormone analysis (mean mass ± SD; 8.2 ± 1.0 g); therefore we only investigated the potential influence of CORT in NOWA and SWTH. For these analyses, we used ANOVAs to compare CORT and T levels in both males and females sampled within 3 minutes of capture in a net (baseline CORT samples) to individuals for whom precise capture time was unknown. This design allowed us to determine whether CORT levels were higher in birds with unknown capture times and whether this influenced circulating T levels as has been indicated in some studies (Deviche et al., 2001; Swett and Breuner, 2008). Finally, we also analyzed the relationship between T and diameter of the cloaca using ANOVA.

3. Results

3.1. Testosterone patterns related to sex, location, and age

There were no significant interactions between location, age and sex on T levels of SWTH and none of these factors had a significant effect on T in SWTH (Table 2; Fig. 2). T levels of NOWA showed no significant interactions between location, age, or sex. There was a significant effect of location on T levels in NOWA but the effects sex and age were not significant (Table 2). Post-hoc pairwise analyses revealed that male NOWA have higher T in Maine ($t = 2.6$, $p = 0.048$; Fig. 2) while T in female NOWA was not different between sites ($t = 0.49$, $p = 0.96$). Further, T in male NOWA in Maine was higher than in female NOWA from both locations but only significantly so for females in Louisiana (Female Maine-Male Maine: $t = 2.5$, $p = 0.07$; Female Louisiana-Male Maine: $t = 2.7$, $p = 0.04$). We found no significant interactions between sampling location, age, or sex on T of MAWA thus first-order effects were investigated. There were significant effects of sex and sampling location on T, but not age (Table 2). While both male and female MAWA had higher T in Maine, as shown by pairwise analyses, the difference is significant in males only (Males: $t = 3.7$, $p < 0.01$; Females: $t = 1.6$, $p = 0.4$; Fig. 2). Additionally female MAWA in Maine had significantly higher T than males in Louisiana ($t = 4.0$, $p < 0.01$; Fig. 2).

3.2. Other factors in relation to testosterone

For SWTH, none of the other variables examined (date, handling time, energetic condition, and time of day) had a significant effect on T (Table 3). There was no effect of energetic condition or time of day on T in NOWA (Table 3). In NOWA, date had a significant and positive effect on T (Table 3). Handling time also had a significant effect on T in NOWA however the 95% confidence interval for the parameter estimate overlapped zero indicating that there is no net effect of handling time. In MAWA, date had a significant positive relationship with T while the other variables tested were not related to T levels (Table 3).

There was no effect of sex on either CORT or T levels between birds sampled within 3 minutes of capture compared to those with unknown capture times (which could have been up to 30 minutes) in either species tested (T, SWTH: $F_{1,22} = 0.37$, $p = 0.55$; T, NOWA: $F_{1,16} = 0.03$, $p = 0.86$; CORT, SWTH: $F_{1,22} = 0.34$, $p = 0.57$; CORT, NOWA: $F_{1,16} = 0.16$, $p = 0.70$). CORT was significantly higher in birds with unknown capture times compared to those sampled within 3 minutes (SWTH: $F_{1,22} = 4.6$, $p = 0.04$; NOWA: $F_{1,16} = 24.6$, $p < 0.001$; Fig. 3). However, there was no significant difference in T between birds sampled within 3 minutes of capture and those with an unknown capture time (SWTH: $F_{1,22} = 0.001$, $p = 0.97$; NOWA: $F_{1,16} = 2.70$, $p = 0.12$; Fig. 3).

Neither the total number of migrants nor the number of migrants in each species' foraging group was related to T at either location for any of the three species studied (Table 4). Diameter of the cloaca was not different between males and females in any of the three species studied (SWTH: $F_{1,151} = 0.39$, $p = 0.54$; NOWA: $F_{1,59} = 0.01$, $p = 0.92$; MAWA: $F_{1,60} = 0.19$, $p = 0.67$). Additionally, cloacal diameter was not related to circulating T levels in any three species (SWTH: $F_{1,151} = 0.55$, $p = 0.46$; NOWA: $F_{1,58} = 1.34$, $p = 0.25$; MAWA: $F_{1,60} = 0.05$, $p = 0.83$).

4. Discussion

4.1. Geographic patterns of testosterone

In all three species studied, males had higher T in Maine, where they are closer to their breeding grounds, compared to Louisiana, although this pattern was not statistically significant in SWTH. This increase in T likely coincides with testis recrudescence which has been shown to occur throughout vernal migration in other passerine species(Bauchinger et al., 2008, 2007; Wingfield and Farner, 1978a, 1978b). Whereas studies of captive birds also indicate that T increases as males progress through simulated vernal migration (Bauchinger et al., 2008; Bluhm et al., 1991; Ramenofsky et al., 1999; Schwabl and Farner, 1989a, 1989b), it should be noted that hormone levels between captive and free-living birds may not be comparable (see Wingfield et al., 1990).

Within songbirds, the extent of overlap between migration and breeding preparation is likely to be driven by the length of the migratory journey such that long-distance migrants, including Nearctic-Neotropical migrants, are expected to have substantial temporal overlap between breeding preparation and vernal migration (Ramenofsky and Wingfield, 2006; Ramenofsky, 2011). Field studies investigating T levels in relation to breeding ground proximity in male migrants have yielded conflicting results. Wingfield and Farner (1978a, 1978b) found that T increases during the migratory period as males approach the breeding grounds in both the long-distance subspecies (*Z.l. gambelii*) and the medium-distance subspecies (*Z. l.*

pugetensis) of the White-crowned Sparrow. Bauchinger and colleagues (2007) did not find an increase in T until Garden Warblers arrived on the breeding grounds. It should be noted that many of their samples were below their assay's detection limit, which may have impeded the ability to detect changes in the T levels of migrating Garden Warblers. Tonra et al. (2013) also failed to find a relationship between androgen levels and distance to the breeding grounds in American Redstarts as they prepared for their vernal migration.

While androgens, including T, are generally considered to be "male" hormones, female vertebrates also have meaningful levels of circulating androgens and possess androgen receptors (Staub and De Beer, 1997). In female birds, T is produced largely by the ovaries but also may be secreted by the adrenal glands, and T levels vary seasonally (Ketterson et al., 2005; Staub and De Beer, 1997). In this study, we did not detect any changes in T levels in female songbirds during spring migration. Wingfield and Farner (1978a) found T to increase during migration in the long-distance migrant subspecies of White-crowned Sparrow but not in their medium distance conspecifics (Wingfield and Farner, 1978a). However in both of these studies by Wingfield and Farner (1978a, 1978b), estradiol was higher in late migrants and upon arrival at the breeding grounds compared to earlier time periods. Once produced, T may be immediately converted into estradiol or may be released into general circulation for direct use or later conversion to estradiol by target tissues (Adkins-Regan, 2005). While we did not measure estradiol in this study because of sampling limitations, one might predict estradiol to increase throughout the migratory period in female songbirds.

In this study we found female MAWA to have relatively high T levels at both locations. In fact, T levels in female MAWA were higher than in male MAWA at our Louisiana sampling location. Outside of the breeding season T levels in songbirds remain fairly low in both sexes, and it is apparently rare for females to have higher T than males in any season (see Ketterson et al., 2005). However, circulating levels of T in male and female Northern Cardinals (*Cardinalis cardinalis*) were similar during the non-breeding season (Devries et al., 2011; Jawor, 2007).

Similarly, T levels in Downy Woodpecker (*Picoides pubescens*) females were as high as or higher than in males during the non-breeding season (Kellam et al., 2004). Both of these species are non-migratory and both sexes may defend territories outside of the breeding season. In contrast, MAWA is a long-distance Nearctic-Neotropical migrant in which males are responsible for most territory defense in the breeding season (Dunn and Hall, 2010). However, other species of wood warblers are known to aggressively defend territories during the non-breeding season (Greenberg et al., 1996; Marra, 2000) and MAWA have been shown to segregate by sex on their wintering grounds (Ornat and Greenberg, 1990). While it is possible that female MAWA sustain high T throughout the year to promote aggressive behaviors related to winter territory defense, given the relatively low T levels seen in male MAWA early in migration and since males likely show aggressive behaviors as well, this potential explanation for female T levels in MAWA warrants more attention.

4.2. Temporal patterns of testosterone

In MAWA and NOWA, T increased with date, which may indicate that the pattern detected between sampling locations is a function of the time since breeding preparation began, rather than the geographic proximity to the breeding grounds. This observed pattern makes sense because after being photostimulated, the HPG axis increases production of hormones including T (Deviche and Small, 2001; Hahn et al., 2009). Vernal migration takes approximately 20 days for long-distance migrants complete (Ewert et al., 2012; Stutchbury et al., 2009; Wikelski et al., 2003). Based on our mixed-effect models, we estimate that circulating T would increase by 1.3 ng/ml in both NOWA and MAWA during that time. Testosterone levels in breeding male songbirds are generally within the 1 – 5 ng/ml range (Ketterson et al., 2005), so this observed within-season increase represents a meaningful change in hormone levels.

4.3. Other factors considered in relation to testosterone

T was not related to time of day, energetic condition, cloacal protuberance, or handling time. Previous studies have found that T follows a diel rhythm with highest levels during the over-night or early morning periods (Goymann and Trappschuh, 2011; Hau et al., 2002; Kempenaers et al., 2008). Given that we only sampled T during daytime hours, it is not surprising that we did not find T to correlate with time since sunrise. Removal of T has been shown to suppress the development of migratory traits including fattening and increases in mass (Deviche, 1995; Schwabl and Farner, 1989b; Stetson and Erickson, 1972) and experimental increases in T have been shown to promote the development of such traits (Owen et al. 2014; Tonra et al. 2011b; 2013). Although understudied, T may also play a role in the regulation of muscle anabolism (see Ramenofsky, 2011) and these seemingly opposing roles of T with regard to condition may explain the lack of relationship in this study. While some studies have indicated T levels may be related to condition changes in preparation for migration (Tonra et al. 2011b; 2013) it is likely that T's role in fattening and other migratory traits is complicated and requires further study (Ramenofsky and Németh, 2014).

The cloacal region is used for sperm storage in male birds and studies have shown that T plays a role in the development of the cloacal protuberance (Ramenofsky and Németh, 2014; Tonra et al., 2011b). Given that the proportions of migrating songbirds that have been found to produce measurable amounts of sperm is low (Quay, 1986, 1985a, 1985b) and that T levels we report in this study are lower than what is typical of breeding songbirds (Ketterson et al., 2005), the lack of relationship between cloacal diameter and T is not unexpected. That said, Tonra et al. (2011a) found an inverse relationship between cloacal diameter and arrival date on the breeding grounds with early arriving males having larger cloacal diameters, higher androgen levels, and better breeding success. In our study, cloacal diameter was not different between males and females indicating that that development of the cloacal protuberance in males had yet to commence.

Time spent in captivity has the potential to influence hormone levels and studies often exercise caution by including handling time in statistical analyses of T and other hormones. However, the effect of relatively short handling times (< 30 min), as in this study, are not likely to significantly influence circulating T (Peters et al., 2001; but see Devries et al., 2011). While we define handling time as the latency between extraction from a net and sampling, precise capture time in a net is unknown for most of the birds in this study. Even though previous studies have suggested that high levels of CORT caused by capture and handling may suppress T and other hormones of the HPG axis (Deviche et al., 2001; Swett and Breuner, 2008), we saw no indication of this in our study. Testosterone levels in birds with elevated "stress" levels or CORT were no different from birds with lower, baseline CORT levels. Our results are not novel, however, since other studies have found similar results (e.g. Devries et al., 2011; Wikelski et al., 1999), but this study does add to a growing body of evidence of the complex relationship between these two hormones. For example, higher CORT levels may increase free (unbound) T (Deviche et al., 2001) and variation among levels of T, CORT, and their shared binding globulin do not seem to influence T's availability to target tissues (Swett and Breuner, 2008).

Testosterone is known to mediate inter- and intra-specific aggression and territorial defense in songbirds during the breeding season (Balthazart, 1983; Wingfield et al., 2001). In this study, however, we found no evidence that T was related to or influenced by competition during migratory stopover as assessed by both the total number of other migrants present and the number of migrants within a species' foraging group. That said, T may influence the rate of and time spent foraging during the breeding season (Lynn et al., 2000) and thus the relationship between T, competition, and foraging activities during migration warrants further study during the migratory periods.

5. Conclusions

The growing consensus is that male migrants increase their T during vernal migration, likely as a result of overlapping life-history stages as breeding preparation begins. While female migrants did not vary during migration in this study, most birds of both sexes had slightly higher T levels than what is found in other Nearctic-Neotropical migrants during the wintering period (e.g. Tonra et al., 2013; Wingfield and Farner, 1978a, 1978b). Additionally, our observed temporal increase in T during migration may reflect the time that has elapsed since photostimulation rather than simply a geographic proximity to the breeding grounds. The overlapping life history stages of breeding and migration seen in this and other studies likely facilitate a smooth transition to commence breeding activities (e.g. territory establishment) immediately upon arrival on the breeding grounds.

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Figure Captions

Fig. 1. Breeding ranges for (A) Swainson’s Thrushes, (B) Northern Waterthrushes, and (C) Magnolia Warblers. Sampling locations in Louisiana and Maine are indicated by filled triangles. Breeding range data were provided by BirdLife-International and NatureServe (2014).

Fig. 2. Circulating testosterone levels in male (circles) and female (triangles) Swainson’s Thrushes, Northern Waterthrushes, and Magnolia Warblers sampled at a southern and a northern site during vernal migration. Data points are back-transformed means and error bars represent ± 1SE.

Fig. 3. Circulating testosterone levels and corticosterone levels in (A) Swainson’s Thrushes and (B) Northern Waterthrushes for bird sampled within three minutes of capture (squares) and those in which precise capture time is unknown (circles). Data points are back-transformed means and error bars represent ± 1SE.

TABLE 1. Number of birds sampled in Louisiana and Maine during spring migration by age and sex.

		Male			Female		
Species	Site	SY	AS	AH	SY	ASY	AHY
Swainson's Thrush	Louisian	57	55	6	57	33	8
	Maine	22	20	2	18	23	1
Northern	Louisian	19	24	17	21	10	5
	Maine	13	19	2	24	19	4
Magnolia Warbler	Louisian	19	20	0	21	6	0
	Maine	34	21	0	29	17	0

TABLE 2. Linear mixed model analysis of the effects of age, sex, and sampling location on testosterone. Model effect estimates and standard error is given for each species and variable. Chi-squared statistics derive from likelihood ratio tests that compared a model with the variable in question to the null model. Asterisks denote statistical significance of a variable.

		Model Statistics		Comparison to Null Model	
Species	Fixed	Estimat	SE	X^2	*P*
Swainson's Thrush	Age	0.89	0.02	0.76	0.39
	Sex	0.008	0.01	0.42	0.52
	Location	0.03	0.02	2.77	0.10
Northern	Age	-0.11	0.14	0.58	0.45
	Sex	0.23	0.13	3.22	0.07
	Location	-0.29	0.14	3.87	0.049*
Magnolia Warbler	Age	-0.17	0.12	1.81	0.18
	Sex	-0.13	0.12	13.16	0.001*
	Location	-0.49	0.13	13.20	0.0003

TABLE 3. Linear mixed model analysis of the effects of energetic condition, handling time, sampling date, and time of day on testosterone. Model effect estimates and standard error is given for each species and variable. Chi-squared statistics derive from likelihood ratio tests that compared a model with the variable in question to the null model. Asterisks denote statistical significance of a variable.

		Model Statistics		Comparison to Null Model	
Species	Fixed Effect	Estimat	SE	X^2	*P*
Swainson’s Thrush	Energetic	0.003	0.002	0.46	0.50
	Handling Time	-0.0009	0.003	0.10	0.76
	Date	0.0004	0.0005	0.65	0.42
	Time of Day	0.003	0.002	0.74	0.39
Northern	Energetic	0.005	0.04	0.01	0.91
	Handling Time	0.07	0.04	5.00	0.03*
	Date	0.01	0.005	5.18	0.02*
	Time of Day	-0.003	0.02	0.02	0.90
Magnolia Warbler	Energetic	0.007	0.07	0.01	0.92
	Handling Time	0.008	0.03	3.69	0.06
	Date	0.02	0.004	10.53	0.001*
	Time of Day	-0.008	0.02	0.25	0.62

TABLE 4. Linear mixed model analysis of testosterone and foraging competition. Testosterone was compared to the total number of other migrants and to the number of migrants within each species’ foraging group (see methods). Statistics derive from likelihood ratio tests that compared a model with the variable in question to the null model.

		Louisiana		Maine	
Species	Comparison	X^2	*P*	X^2	*P*
Swainson’s Thrush	Total	0.26	0.61	1.52	0.22
	Foraging Group	0.05	0.82	1.13	0.29
Northern Waterthrush	Total	0.33	0.57	3.30	0.07
	Foraging Group	0.14	0.71	1.14	0.29
Magnolia Warbler	Total	0.33	0.57	0.14	0.71
	Foraging Group	0.37	0.54	0.35	0.55

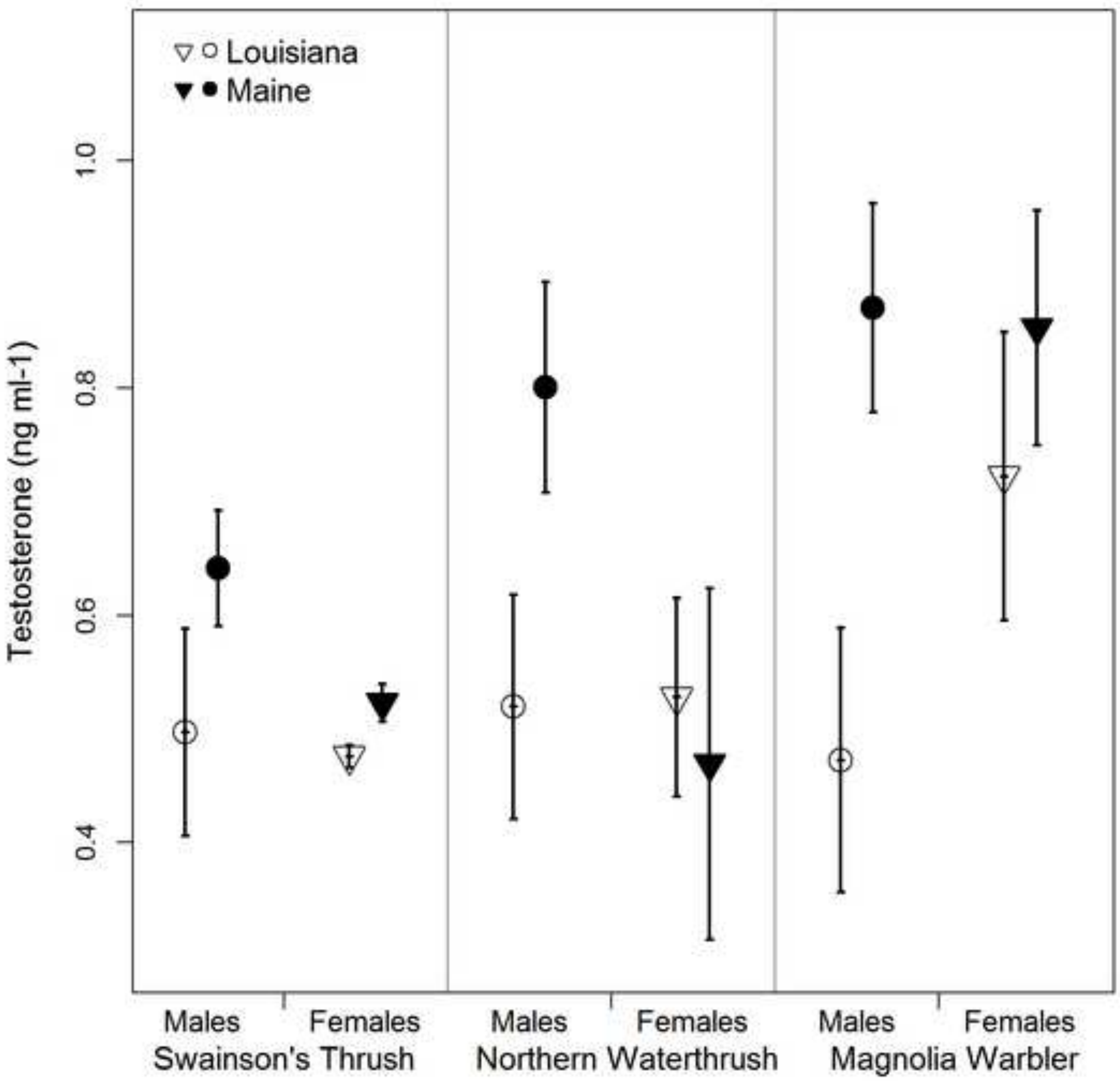
Figure 1

A. SWTH

B. NOWA

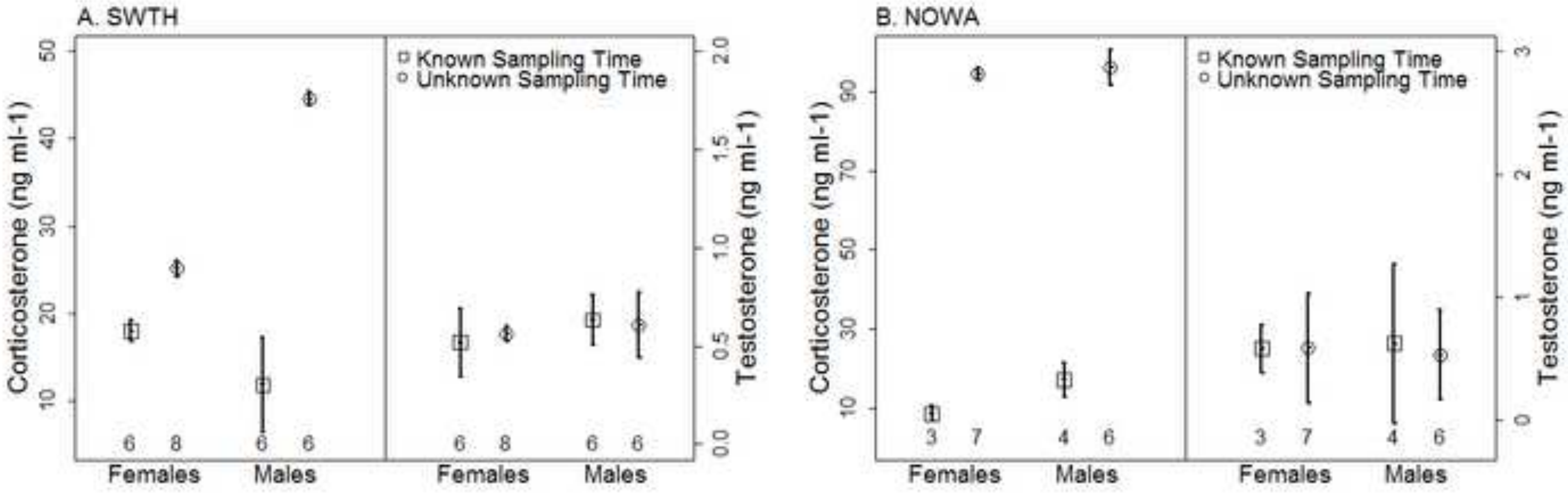
C. MAWA

Figure 2



▽ ○ Louisiana
▼ ● Maine
Testosterone (ng ml-1)
1.0
0.8
0.6
0.4
Males
Females
Swainson's Thrush
Males
Females
Northern Waterthrush
Males
Females
Magnolia Warbler

Figure 3


A. SWTH
Corticosterone (ng ml-1)
10
20
30
40
50
6
8
6
6
Females
Males
□ Known Sampling Time
○ Unknown Sampling Time
6
8
6
6
Females
Males
Testosterone (ng ml-1)
0.0
0.5
1.0
1.5
2.0
B. NOWA
Corticosterone (ng ml-1)
10
30
50
70
90
3
7
4
6
Females
Males
□ Known Sampling Time
○ Unknown Sampling Time
3
7
4
6
Females
Males
Testosterone (ng ml-1)
0
1
2
3